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Analysis of intracellular doxorubicin and its metabolites by ultra-high-performance liquid chromatography

Kumiko Sakai-Kato*, Eiko Saito, Keiko Ishikura, Toru Kawanishi

Division of Drugs, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

Doxorubicin, a highly effective anticancer drug, produces severe side effect such as cardiotoxicity, which is mainly caused by its metabolite, doxorubicinol. While *in vitro* studies by measuring cellular concentration of doxorubicin have been reported, there have been no reports on measuring cellular concentration of the metabolites. In this report, we developed a sensitive and high-throughput method for measuring cellular concentrations of doxorubicin and its metabolites by ultra-high-performance liquid chromatography. The method achieved more than 96% recovery of doxorubicin and its metabolites from cell homogenates. Using simple separation conditions, doxorubicin and its three main metabolites, and the internal standard, were separated within 3 min. The method has a limit of quantification of 17.4 pg (32.0 fmol) injected doxorubicin. This high sensitivity enables the detection and intracellular quantification of doxorubicin and its metabolite, doxorubicinol, in cell homogenates, and its use will facilitate studies of the relationship between doxorubicin pharmacokinetics and therapeutic outcome.

1. Introduction

The anthracycline doxorubicin, which was originally produced by *Streptomyces peucetius* var. *caesius*, is one of the most widely used anticancer agents, and it has a broad spectrum of activity against a variety of malignancies [1,2]. However, the clinical use of doxorubicin is limited by the side effect of cumulative dose-dependent irreversible chronic cardiomyopathy by doxorubicin and its metabolite, and optimal dose schedules remain a matter of debate [3]. *In vitro* studies have demonstrated a relationship between intracellular doxorubicin levels and cytotoxicity [4,5]. It was proposed that monitoring of intracellular doxorubicin concentrations could help elucidate the relationship between anthracycline pharmacokinetics and therapeutic outcome [6]. Although doxorubicinol, which is one of the major metabolites, has more potent cardiotoxic action than doxorubicin [3], there have been no reports on measuring intracellular level of doxorubicinol, probably due to the detection sensitivity. In this report, we developed a method for measuring intracellular concentrations of doxorubicin and its metabolites.

A number of methods for the simultaneous quantification of doxorubicin and its metabolites in biological samples are based on high-performance liquid chromatography (HPLC) with fluorescence detection [7–11]. Efforts to quantify anthracycline drugs in blood and tissues have encountered methodological difficulties, possibly because of a combination of failure to achieve chromatographic resolution of the various metabolites and the high affinity of these drugs for cellular constituents [12].

Ultra-high-performance liquid chromatography (UHPLC) is a new category of separation techniques that is based upon well-established principles of liquid chromatography. The resolution, sensitivity, and speed of analysis are dramatically increased by the use of 2-μm particles in the stationary phase, high linear velocities for the mobile phase, and instrumentation that operates at higher pressures than those used in HPLC [13–15].

Because doxorubicin intercalates into DNA, to achieve good recovery we used two enzymes during sample preparation that are commonly employed in the purification and degradation of DNA. By using UHPLC, we developed a simple and high-throughput method with high sensitivity for the analysis of intracellular doxorubicin and its metabolites.

2. Materials and methods

2.1. Drugs and chemicals

Doxorubicin hydrochloride and daunorubicin hydrochloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka,

Abbreviations: UHPLC, ultra-high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; Triton X-100, polyoxyethylene(10) octylphenyl ether; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

* Corresponding author. Tel.: +81 3 3700 9662; fax: +81 3 3700 9662.
E-mail address: kumikato@nihs.go.jp (K. Sakai-Kato).

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Japan). Doxorubicinol hydrochloride and doxorubicinone were purchased from Toronto Research Chemicals Inc. (North York, Canada). Doxorubicinolone was synthesized from doxorubicinol by acidic hydrolysis (0.5 N HCl) at 50 °C for 24 h. Aglycone was extracted with chloroform by a liquid–liquid extraction method [16].

DNase I, phenylmethylsulfonyl fluoride (PMSF), proteinase K, and zinc sulfate heptahydrate were obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). Polyoxyethylene(10) octylphenyl ether (Triton X-100), magnesium chloride, sodium dihydrogen phosphate dehydrate, and phosphoric acid were obtained from Wako Pure Chemical Industries, Ltd. HPLC-grade isopropanol, HPLC-grade acetonitrile, and HPLC-grade methanol were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Cell culture

HeLa cells (Health Science Research Resources Bank, Osaka, Japan) and HT29 cells (American Type culture Collection, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., CA, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan) and 100 U/mL penicillin–streptomycin mixture (Invitrogen). Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

2.3. Preparation of samples for HPLC

Cells were washed with PBS, resuspended in 300 μL PBS and lysed on ice with an ultrasonic homogenizer (Astrason, Misonix Inc., IN, USA). The lysed samples were treated with enzymes according to the method of Anderson et al. [4]. Five microliters Triton X-100 (5%) and 5 μL proteinase K (10 mg/mL) were added to an aliquot of 200 μL cell homogenates. After brief mixing, the samples were incubated for 1 h at 65 °C in a water bath. An aliquot of 2.5 μL PMSF (10 mM in isopropanol) was added and the samples were incubated for 10 min at room temperature. Then 5 μL $MgCl_2$ (0.4 M) and 10 μL DNase I (1 mg/ml) were added and the samples were incubated in a water bath at 37 °C for 30 min.

Each 225 μL sample was then mixed with 225 μL methanol and 22.5 μL $ZnSO_4$ (400 mg/mL) and centrifuged at 15,000 × *g* for 5 min in a microcentrifuge (Model 3740, Kubota Corp., Tokyo, Japan); the supernatants were then collected. A 30-μL aliquot of each supernatant was mixed with 5 μL of the internal standard (daunorubicin, 10 μg/mL in methanol), 50 μL ice-cold methanol and 15 μL Milli-Q water, and filtered through a 0.20-μm filter (Millex-LG, Millipore Corp., Tokyo, Japan). The filtrates were transferred to autosampler vials before UHPLC analysis.

The amounts of protein in cell homogenates were determined using BIO-RAD protein assay reagent (BIO-RAD, CA, USA).

2.4. HPLC apparatus

High-throughput quantification of doxorubicin and its metabolites was performed using a Hitachi LaChrom ULTRA system, equipped with an L-2160U pump, an L-2200U automated sample injector, an L-2300 thermostatted column compartment, and an L-2485U fluorescence detector (Hitachi, Tokyo, Japan).

2.5. Chromatographic conditions

Samples were analyzed on a Capcell Pak C18 IF column (2.0 × 50 mm; particle size, 2 μm; Shiseido Corp., Tokyo, Japan). The mobile phase consisted of a 50-mM sodium phosphate buffer (pH 2.0): acetonitrile mixture (65:27 v/v). The mobile phase was delivered at a rate of 300 μL/min and the column temperature was maintained at 25 °C. The fluorescence detector was operated at an excitation wavelength of 470 nm and an emission wavelength of 590 nm. A volume of 5 μL of sample was injected each time.

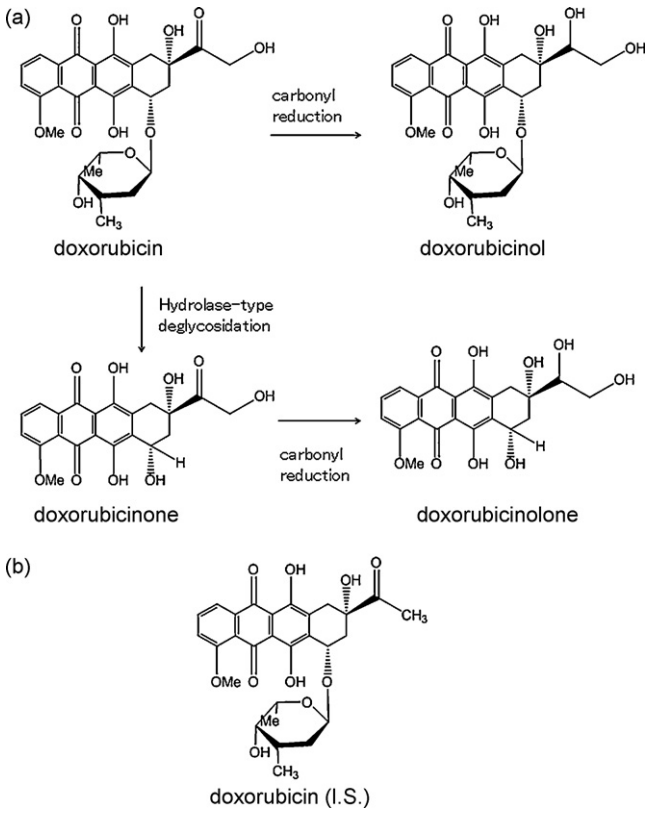

Fig. 1. Schematic showing the chemical structure of doxorubicin and its metabolites (a) and the chemical structure of daunorbicin, the internal standard (b).

2.6. Confocal analysis of live cells

The intracellular distribution of doxorubicin was examined by live-cell confocal microscopy (Carl Zeiss LSM 510, Germany). Dedicated software supplied by the microscope manufacturers was used to collect data, and images were exported as TIFF files. HeLa cells (1.5×10^5) were plated into 35-mm glass-bottomed dishes coated with poly-L-lysine (Matsunami, Osaka, Japan) and cultured in DMEM containing 10% FBS and 100 U/mL penicillin–streptomycin mix. After 2 days of incubation (37 °C, 5% CO_2), the culture medium was replaced and the cells were exposed to 1 μg/mL doxorubicin. After 1 h, cells were washed and kept in Hanks's Balanced Salt Solution (Invitrogen) for subsequent imaging by confocal microscopy.

3. Results and discussion

3.1. Chromatograms

Fig. 1a shows the chemical structure of doxorubicin and the doxorubicin metabolites that were studied in this report, and the structure of the internal standard (daunorubicin) (Fig. 1b). Because these chemicals show native fluorescence, they can be sensitively analyzed by the detection of this fluorescence. Fig. 2 shows the chromatograms resulting from the analysis of a standard solution of doxorubicin, doxorubicinol, doxorubicinolone, doxorubicinone, and the internal standard. All compounds were separated within 3 min with good resolution owing to the use of UHPLC. The pressure was 26.6 MPa at a flow rate of 300 μL/min, but the pressure was not high enough to adversely affect the stability of the column. High repeatability of analyte retention times was achieved; the relative

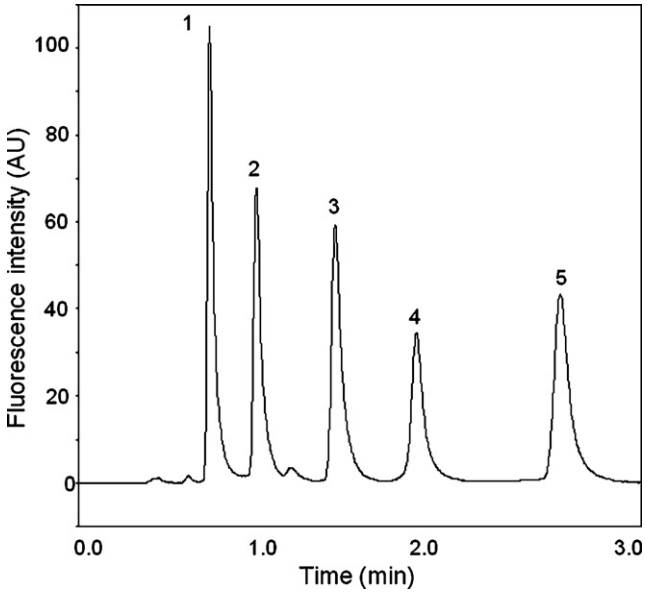

Fig. 2. Chromatogram of doxorubicin and its metabolites. The chromatographic conditions are described in Section 2. 1, doxorubicinol; 2, doxorubicin; 3, doxorubicinolone; 4, daunorubicin (internal standard); 5, doxorubicinone.

standard deviation (R.S.D.) of each peak was less than 0.13% ($n = 5$), for each analyte, at a concentration of 50 ng/mL. The conditions of separation of doxorubicin and its three metabolites were very simple in that the elution was isocratic, and the mobile phase consisted of only two different solvents, whereas some reported methods require three different solvents [7,8]. Fig. 3a is a chromatogram of a homogenate from untreated HeLa cells, and Fig. 3b is a chromatogram of an equivalent homogenate spiked with doxorubicin and its metabolites at a concentration of 500 ng/mL. No interfering peaks were observed, and doxorubicin, the three metabolites, and the internal standard separated well. These results show that the separation conditions were optimized with selectivity to each compound.

3.2. Detection limits and quantitation limits

Detection limits and quantitation limits of doxorubicin and its metabolites were determined based on the signal-to-noise approach, (S:N ratio, 3:1 for detection limits and 10:1 for quantitation limit) (Table 1). The quantitation limits of doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone ranged between 11.7 and 24.5 pg/injection. The quantitation limit of 17.4 pg/injected doxorubicin (32.0 fmol/injected doxorubicin) was about 2 times lower than the limit ever reported using conventional HPLC [8], and more than 10 times lower than other reported values [7,9–11]. We suggest that the high resolution and sensitivity of UHPLC are responsible for this improvement.

Table 1
Detection limits and quantitation limits of doxorubicin and its metabolites.

Compound	Detection limit (pg/injection)	Quantitation limit (pg/injection)
Doxorubicin	5.2	17.4
Doxorubicinol	3.5	11.7
Doxorubicinolone	6.0	19.8
Doxorubicinon	7.4	24.5

The detection and quantitation limits of doxorubicin and its metabolites were determined based on signal-to-noise ratios (3:1 for detection limits, and 10:1 for quantitation limits).

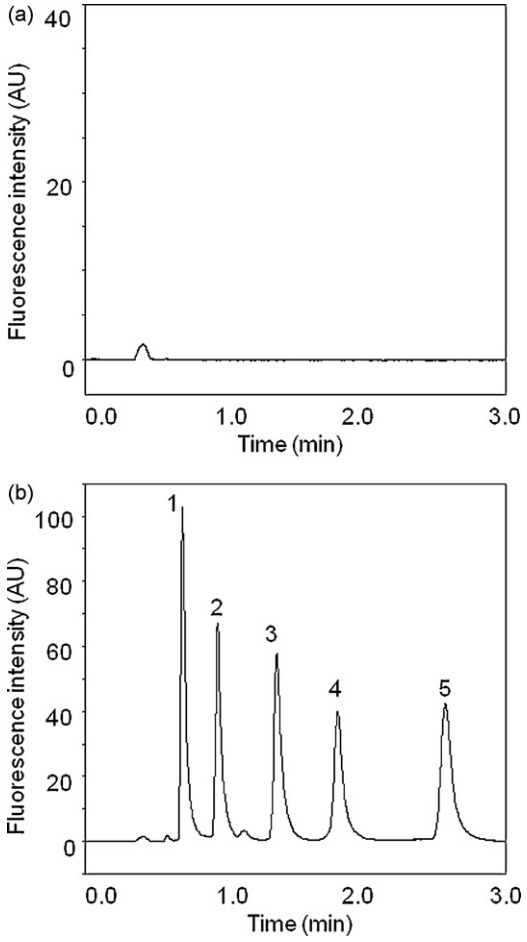

Fig. 3. Chromatograms of (a) HeLa cell homogenate and (b) HeLa cell homogenate spiked with doxorubicin and its metabolites. The chromatographic conditions were the same as in Fig. 2 and are described in Section 2. 1, doxorubicinol; 2, doxorubicin; 3, doxorubicinolone; 4, daunorubicin (internal standard); 5, doxorubicinone.

3.3. Drug recovery

Doxorubicin and its metabolites have a high affinity for cellular constituents [12]. Confocal fluorescence imaging of a HeLa cell that was exposed to doxorubicin for 1 h showed that doxorubicin had preferential affinity for the nucleus (Fig. 4). Drug recovery was assessed by adding doxorubicin, doxorubicinol, doxorubicinolone,

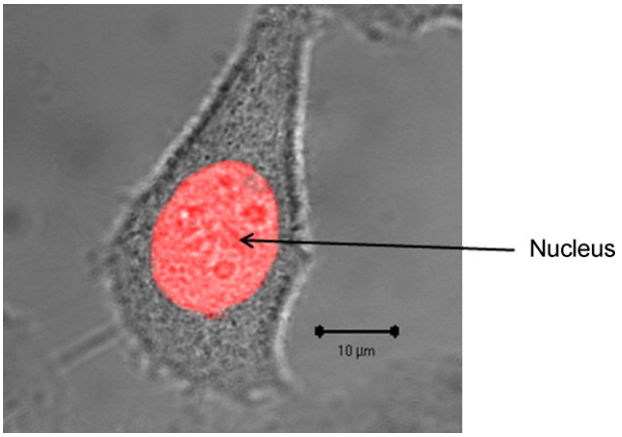

Fig. 4. Intracellular distribution of doxorubicin. HeLa cells were exposed to 1 μg/mL doxorubicin for 1 h, washed, and observed by confocal microscopy. Scale bar: 10 μm.

Table 2
The recovery of doxorubicin and its metabolites from HeLa cell homogenates.

Compound	Recovery rate	
	(%)	R.S.D. (%)
Doxorubicin	102	3.3
Doxorubicinol	105	2.9
Doxorubicinolone	96	1.4
Doxorubicinone	98	2.1

Each compound (1 μg/mL) was added to HeLa cell homogenates, which were then treated as described in Section 2. Mean values for percentage recovery are given ($n = 3$).

and doxorubicinone to homogenates of two representative human cancer cell-lines, HeLa cells (derived from human epithelial carcinoma) and HT29 cells (derived from human colon adenocarcinoma grade II) before sample preparation. When the cell homogenate was treated with only methanol and $ZnSO_4$, by a method previously used to study doxorubicin and its metabolites in plasma [9], the percentage recoveries of doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone were 84.1 ± 8.2%, 63.2 ± 3.0%, 78.4 ± 3.7%, and 88.7 ± 2.7%, respectively, ($n = 3$). Doxorubicinol is harder to recover because it has higher affinity to the cellular constituents, and this affinity to the cellular constituents causes its cytotoxicity [3]. To obtain a higher drug recovery, the cells were lysed by an ultrasonic homogenizer, and the cellular proteins were further digested and solubilized with a combination of Triton X-100 and the endopeptidase proteinase K. Nuclear DNA was hydrolyzed by treatment with DNase I in the presence of divalent cations. Using these enzymatic treatments in accordance with the method of Anderson et al. [4], the recovery dramatically improved (Table 2). Furthermore, for each compound, a satisfactory within-day repeatability was achieved with R. S. D. of ≤ 3.3%, $n = 3$.

3.4. Linearity of the calibration plots

We created calibration plots for doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone (Fig. 5). The *y*-axis is the ratio of the peak area of each analyte tested to the peak area of the internal standard (daunorubicin), and the *x*-axis is the concentra-

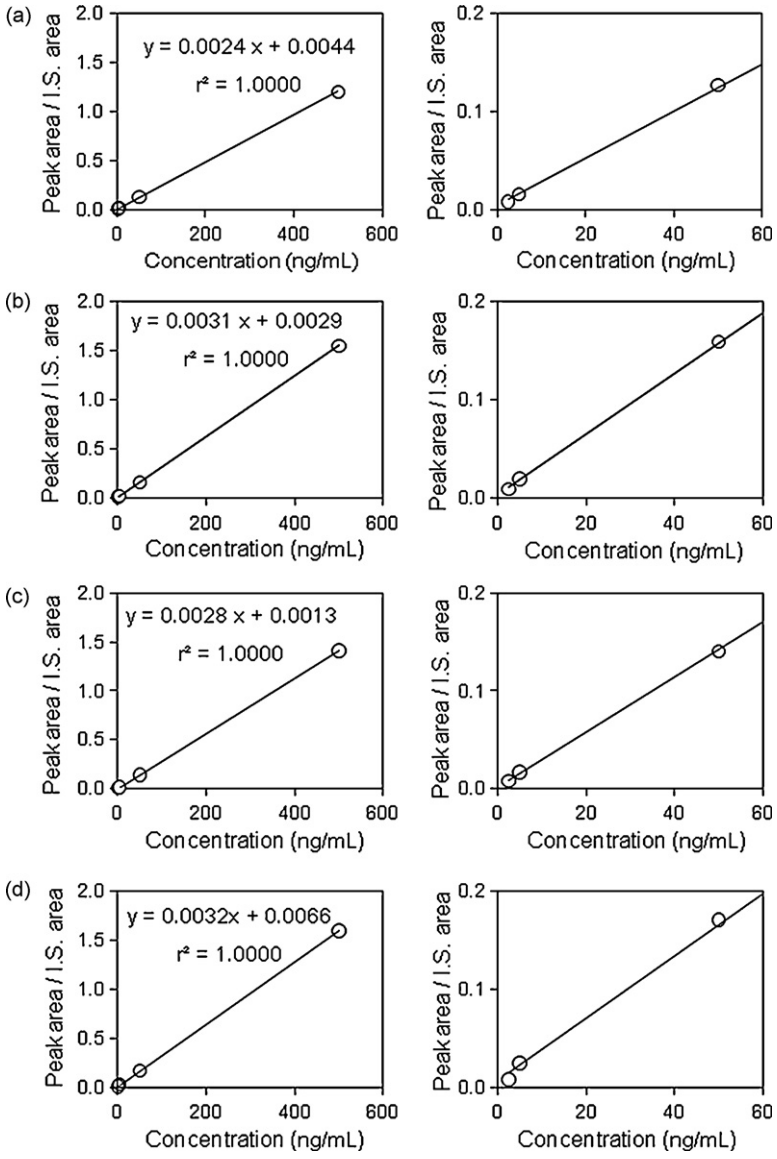

Fig. 5. Linearity of the calibration curves of doxorubicin and its metabolites. Calibration plots for (a) doxorubicin, (b) doxorubicinol, (c) doxorubicinolone, and (d) doxorubicinone. The left and right panels show results for the same HPLC run, but the plots in the right panel focus on the lower concentration ranges. I.S. denotes internal standard.

Table 3
Quantitation of doxorubicin and doxorubicinol in homogenates prepared from doxorubicin-treated cells.

Sample	Doxorubicin (μg/mg cell protein)	Doxorubicinol (μg/mg cell protein)
HeLa cells	3.4 ± 0.55	0.057 ± 0.0060
HT29 cells	3.5 ± 0.38	0.048 ± 0.0029

Cells were treated for 2 h with 10 μg/mL doxorubicin, and cell homogenates were prepared and analyzed, as described in Section 2. Values are given as mean ± S.D. (n (dish number) = 3).

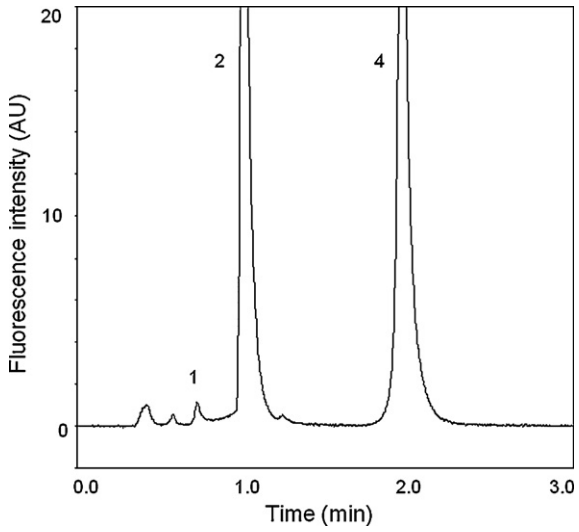

Fig. 6. Chromatogram of cell homogenate obtained 2 h after administration of doxorubicin. HeLa cells were exposed to 10 μg/mL doxorubicin for 2 h. Cell homogenates were then prepared as described in Section 2. 1, doxorubicinol; 2, doxorubicin; 3, daunorubicin (internal standard).

tion of the corresponding analyte. The plots were linear over a wide range of concentrations (r^2 = 1.0; Fig. 5).

3.5. Quantitative determination of the levels of doxorubicin and its metabolite in cells

The validated method described above was used for the simultaneous determination of doxorubicin and its metabolites in human cancer cell-lines. HeLa cells and HT29 cells were exposed to 10 μg/mL doxorubicin for 2 h, and then washed with PBS. Chromatograms of the cell homogenates were obtained by UHPLC (Fig. 6). As shown, doxorubicin and one of its metabolites, doxorubicinol, were detected in cell homogenates (Fig. 6). The results of quantitative determination of doxorubicin and doxorubicinol are shown in Table 3. Values are expressed as amounts per 1 mg cellular protein in each dish, due to the diversity of cell numbers for different dishes, and values are given as mean ± S.D. for three dishes of the same cell type. The S.D. values for different dishes of the same cell type were acceptable. Doxorubicinol is produced by cytosolic carbonyl reductase through the NADPH-dependent aldo-keto reduction of a carbonyl moiety in doxorubicin [17]; our results first demonstrated that both human cancer cell-lines, HeLa cells and HT29 cells, produced doxorubicinol from doxorubicin. In contrast, our results showed that deglycosidation at the daunosamine sugar in doxorubicin, which produces doxorubicinone and doxorubicinolone (Fig. 1a) [17], was negligible in these cancer cell-lines (Fig. 6).

New formulation technologies that aim to enhance the effectiveness and safety of anticancer drugs are currently being developed. For instance, long-circulating and sterically stabilized liposomes containing doxorubicin can markedly increase tumor-specific deposition of drugs and have been approved as clinical products [18]. Other carrier systems such as polymer micelles [19,20] are also being developed for use with doxorubicin. In these technologies, effective release of doxorubicin from the carrier into the target cells is important for effectiveness and safety. Direct quantification of the metabolites may facilitate the assessment and comparison of doxorubicin release from carriers, since it is presumed that metabolism of doxorubicin takes place only after its release from the carriers. Analytical methodology that enables the rapid quantification of doxorubicin and its metabolites, particularly in targeted tumor cells, will facilitate the optimization of carrier-based strategies for doxorubicin delivery and will help provide insight into the toxicity and bioavailability of doxorubicin incorporated into carriers such as liposomes or polymer micelles.

4. Conclusions

Our results show that this methodology provides a significant reduction in analysis time and a considerable increase in assay sensitivity. We demonstrated that the method is sensitive enough to quantify the levels of doxorubicin and its metabolite within cells, and we predict that it will greatly facilitate studies of doxorubicin pharmacokinetics and clarify the effect of doxorubicin metabolism on therapeutic outcome at the cellular level. Furthermore, this method can be applied to the evaluation of emerging formulation technologies that are based on encapsulated doxorubicin.

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